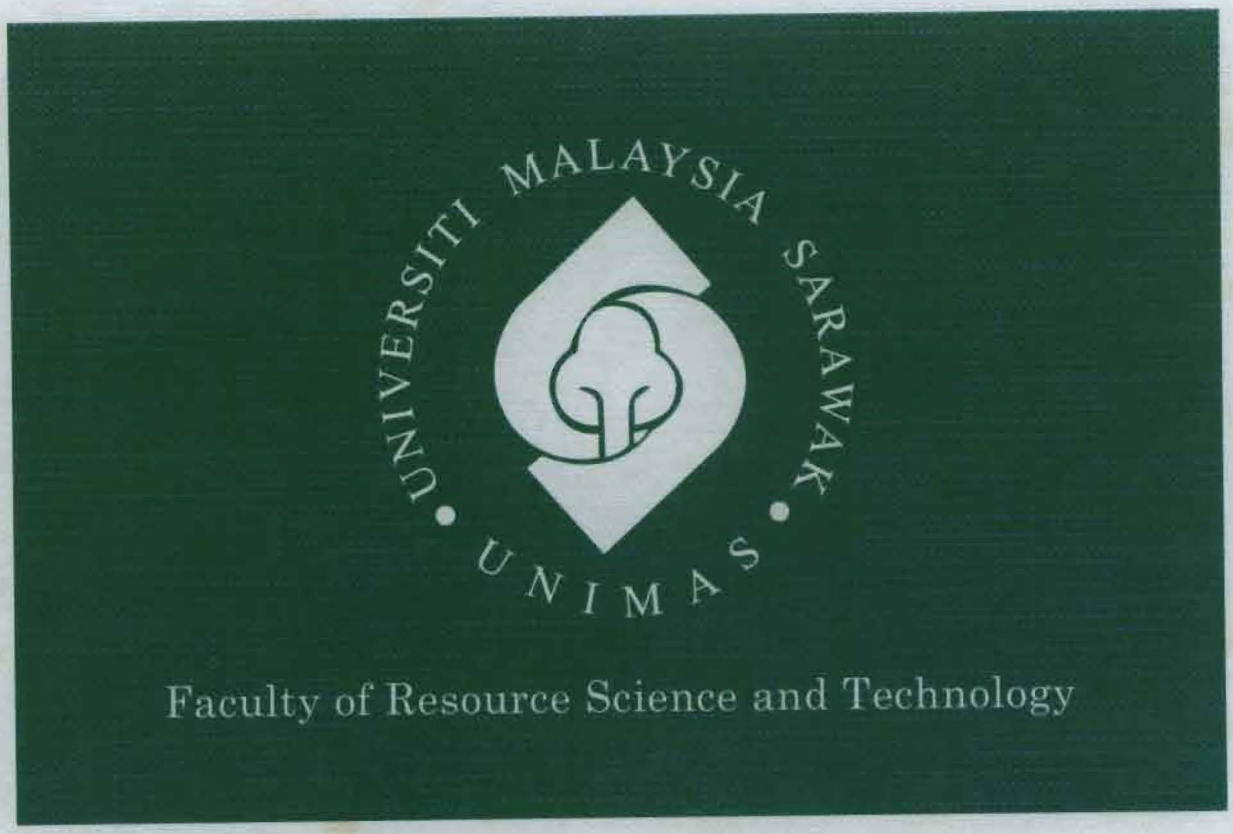

SOLID STATE FERMENTATION OF SAGO WASTE FOR BIOCONVERSION AS LIVESTOCK AND AQUACULTURE FEED

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Bachelor of Science with Honours
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Solid State Fermentation of Sago Waste for Bioconversion as Livestock and Aquaculture Feed

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A Thesis submitted for partial fulfillment of
The requirements for the degree of Bachelor of Science with Honours
(Resource Biotechnology Programme)

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List of Abbreviations

m	Meter
mm	Milimeter
µL	Micro liter
mL	Mililiter
g	Gram
mg	Miligram
M	Molar
v/v	Volume to volume
w/v	Weight per volume
°C	Degree Celcius
%	Percentage
Min	Minute
Rpm	Revolutions per minute
SSF	Solid-state Fermentation
SW	Sago waste
PDA	Potato Dextrose Agar
LB	Luria Broth
DNS	Di-nitrosalicyclic
BSA	Bovine Serum Albumin
UV	Ultraviolet
Mg_2SO_4	Magnesium Sulphate
KH_2PO_4	Monopotassium Phosphate
H_2SO_4	Sulphuric Acid
OD	Optical Density

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Solid State Fermentation of Sago Waste for Bioconversion as Livestock and Aquaculture Feed

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ABSTRACT

Bioconversion of sago waste to value added product is of great interest. Solid substrate fermentation is one of the method of choice for this purpose as it stimulates the natural environment of most microorganisms especially fungi. In this study, lignocellulosic sago waste was treated using *Aspergillus niger* PAN1, *Bacillus amyloliquefaciens* UMAS1002 and *Bacilllus licheniformis* P7 in order to improve its quality as animal feed. The aims of this study are to develop and evaluate the animal feed produced from sago waste via solid substrate fermentation in terms of its fibre content. Five physiochemical analyses were conducted in order to characterize the sago wastes components which are cellulose, hemicelluloses, lignin, crude protein and nitrogen content. The cellulose and hemicelluloses shows a decrease in percentage while the crude protein and nitrogen content increased. The cellulase and xylanase enzymes produced were extracted and assayed using DNS method in order to determine the optimum enzymes activities.

Key words: Solid substrate fermentation (SSF), sago waste, *Aspergillus niger* PAN1, animal feed, bioconversion

ABSTRAK

Penukaran hampas sagu kepada produk nilai tambah adalah amat menarik. Fermentasi substrat pepejal merupakan salah satu kaedah untuk penukaran hampas sagu kerana ianya menyamai proses yang berlaku di dalam persekitaran secara semulajadi dan melibatkan kebanyakan mikroorganisma terutamanya kulat. Di dalam kajian ini, hampas sagu telah dirawat menggunakan Aspergillus niger *PAN1*, Bacillus amyloliquefaciens *UMAS1002 dan* Bacilllus licheniformis *P7 untuk membantu meningkatkan kualiti hampas sagu sebagai makanan ternakan. Tujuan kajian ini adalah untuk menghasilkan dan menilai makanan ternakan yang terhasil daripada hampas sagu melalui kaedah fermentasi pepejal substrat dan menilai kandungan fibernya. Lima analisis fisiokimia telah dilakukan untuk mencirikan komponen hampas sagu, iaitu selulose, hemiselulose, lignin, protein dan kandungan nitrogen selepas proses fermentasi. Kandungan selulose dan hemiselulose didapati menunjukkan pengurangan mengikut peratusan, manakala peratusan protein dan nitrogen pula meningkat jika dibandingkan dengan hampas sagu tidak dirawat sebagai kawalan. Enzim selulase dan xilanase yang dihasilkan juga diekstrak dan dianalisa dengan menggunakan ujian DNS untuk mengenal pasti aktiviti optimum enzim.*

Kata kunci: *Fermentasi substrat pepejal,* Aspergillus niger *PAN1, makanan ternakan, hampas sagu, penukaran.*

1.0 Introduction

1.1 General Introduction

The residues refuse and wastes of agricultural industries constitute a significant proportion of worldwide agricultural productivity, which is estimated to amount to over 30% (Ugwuanyi *et al.,* 2009). One of the agricultural residues that are widely available in Sarawak is the sago wastes. Sarawak is the largest sago-growing areas in Malaysia, which is now the world's biggest sago exporter, annually exporting about 25,000-40,000 tons of sago products to Peninsular Malaysia, Japan, Taiwan, Singapore and other countries (Singhal *et al.*, 2008).

These wastes from the sago industry represent valuable biomass and potential solutions to solve problems of animal nutrition if appropriate technique can be employed for protein enrichment. Protein enrichment of wastes for use in animal nutrition offers opportunities for the reuse of abundant sago waste. Technique available for protein enrichment of these wastes include solid substrate fermentation, ensiling and high solid/slurry processes (Ugwuanyi *et al.,* 2009).

Solid substrate fermentation simulates the living conditions of filamentous fungi in their natural habitat. Therefore, filamentous fungi are more suitable for cultivation in SSF (Santos *et al.*, 2004). It is therefore convenient to grow them in such environment. SSF has been proposed as the suitable pre-treatment of agroindustrial wastes that could allow their use as animal feed. The utilization of agroindustrial wastes as non-inert support in SSF makes the whole process much more economical (Pandey *et al.*, 2000a; Pandey *et al.*, 2000b). In addition, SSF is also a suitable technique for obtaining useful addictives for feed or reagents employed in food manufacture.

The bioconversion of sago waste to value added product for livestock and aquaculture feed application is of great interest. Raw material, which is in this case the

sago wastes, will be upgraded in order to improve the nutritional value and also its palatability. The reagents for feed processing include cellulase, xylanase and mannanase. *Aspergillus niger* PAN1 in combination with *Bacillus amyloliquefaciens* UMAS1002 and *Bacillus licheniformis* P7 will be employed as the microorganisms that will treat the sago wastes due to their abilities to produce the corresponding enzymes. (Duru and Uma 2003a; Duru and Uma 2003b) have demonstrated the potential of using SSF to achieve over 50% increase in the protein content of cocoyam process waste using *A. oryzae.* The protein enriched waste could be used for the feeding of both ruminants and monogastric animals.

Aquaculture feeds require high protein level and low fibre. A study by Iluyemi in 2006 suggested that the limited use of palm kernel cake in fish feeds, due to its high fibre and low protein content, could be overcome by processing the material under SSF conditions with some fungal strains (i.e. *Trichoderma longiobrachiatum*). This treatment leads to significant increase in protein level, and a decrease in cellulose and hemicellulose (Iluyemi *et al.*, 2006). SSF increases the level of protein and decreased cellulose and hemicelluloses due to the action of xylanases and cellulases (Yang *et al.*, 2001).

The bioconversion of sago waste by *A. niger* PAN1, *B. amyloliquefaciens* UMAS1002 and *B. licheniformis* P7 using SSF will be evaluated in this research study.

1.2 Objectives

The aim of this research project is to develop and evaluate the animal feed produced from sago waste via solid substrate fermentation, the potential of treated sago waste to be used as animal feeds and its silage inclusion into diets for livestock and aquaculture.

In order to achieve the aim the specific objectives are:

i. To develop the microbial formulation for bioconversion of sago waste into animal feed.

ii. To perform the SSF of sago waste using the developed microbial formulation.

iii. To determine the cellulolytic enzyme activities produced during the fermentation process.

iv. To characterize the fermented sago waste in term of fibre content, crude protein, nitrogen content, cellulose, hemicelluloses and residual lignin.

2.0 Literature review

2.1 *Aspergillus niger*

The genus *Aspergillus* is geographically found worldwide and consists of more than 180 officially recognized species, and comprises of important group of filamentous ascomycete species, among them are *Aspergillus niger* (Ward *et al.,* 2006). *A. niger* produces colonies that consists of a compact white or yellow basal felt covered by a dense layer of dark brown to black conidial heads (Fungal Genomics Project, 2005). The species is biseriate (Figure 1), in which it is the vesicles that produces sterile cells known as metulae that support the conidiogenous phialides. Plus, conidiophores of *A. niger* are typically 900-1600 μm long, smooth-walled and terminate in pale-brown colored globose vesicles 40-60 μm in diameter.

A. niger is on the Generally Recognized as Safe (GRAS) list of the Food and Drug Administration (FDA) in the United States (Ward *et al.,* 2006). According to Schuster (2002), *A. niger*, like other filamentous fungi, should be treated carefully in order to avoid the formation of spore dust. However, compared to other filamentous fungi, *A. niger* does not stand out as a particular problem concerning allergy or mycopathology (Schuster *et al.*, 2002). According to Ugwuanyi (2009), the use of organisms with GRAS status will help to improve confidence in the final product derived from solid substrate fermentation and ensiling.

SSF is an attractive method for xylanase production, and a large number of different *Aspergillus* species have been reported as good xylanase producers (Beg *et al.*, 2001; Betini *et al.*, 2009). A strain of *A. niger* was isolated from Mexican copra paste and has proved to produce a variety of cell wall degrading enzymes using different substrates upon SSF (Regalado *et al.*, 2000).

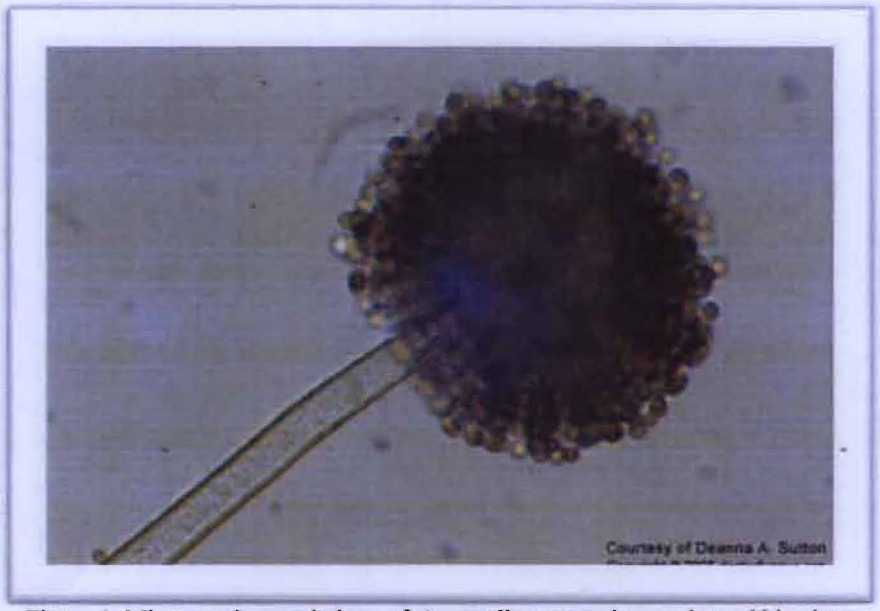

Figure 1: Microscopic morphology of *Aspergillus niger* : image showed biseriate fruiting head. Image retrieved from http://www.doctorfungus.org/thefungi/Aspergillus_niger.php

2.2 *Bacillus* spp.

Bacillus spp. is a gram-positive and rod-shaped bacterium that differentiates into heat resistant endospores under aerobic conditions. *Bacillus* spp. can be generally found in nature; most are saprophytic and are isolated as contaminants. The morphologies of *Bacillus* spp. includes large and spore-forming (Figure 2).

Bacillus spp. has the ability to produce significant amounts of extracellular mannanase. A study by Hossain in 1996 suggested that *Bacillus* sp. KK01 can produced multiple forms of β-mannanases. *B. subtilis* has also reported to be mannanase producers (Zakaria *et al.*, 1998), and is recommended due to its safety, fast growth and ability to secrete high level of mannanase into the medium.

Figure 2: Microscopic morphology of *Bacillus* spp.: image showed gram-positive spore-formers *Bacillus* spp. Image retrieved from http://www.austincc.edu/kotrla/

2.3 Sago waste

Lignocellulosic wastes refer to plant biomass wastes that are composed of cellulose, hemicelluloses and lignin (Mtui, 2009) (Figure 3). Cellulose is a linear polymer that is composed of D-glucose subunits linked by β-1,4 glycosidic bonds forming the dimer cellobiose (Sanchez, 2009). Hemicelluloses, a polysaccharide with a lower molecular weight than cellulose, can be classified into either xylans, mannans, arabinogalactans or arabinans based on their sugar backbone composition. Sago waste will be employed as principle raw materials as carbon source, for the fermentative production of feed related products such as enzymes. The use of lignocellulosic waste such as sago waste in animal feeding is constrained by very low protein content and its limited digestibility and palatability to ruminants (Ugwuanyi *et al.*, 2009). However, they may be applied for animal nutrition following protein enrichment by using a variety of micro and macro fungi and bacteria. The production of protein enriched lignocellulosic waste has been associated

with the reduction in the content of lignocelluloses (associated with loss of biomass via microbial respiration as CO_2) (Ugwuanyi *et al.*, 2009).

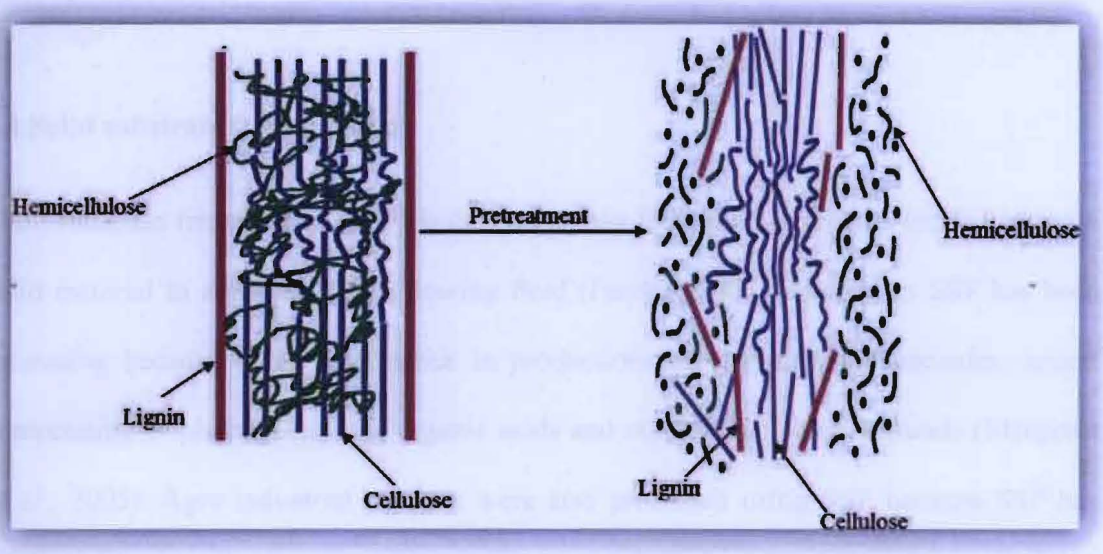

Figure 3: Schematic of the role of pretreatment in the conversion of lignocellulosic biomass (Adapted from Kumar *et al.*, 2009)

According to Sanchez (2009), the major component of lignocellulosic material is cellulose, followed by hemicelluloses and lignin. Cellulose appears in nature to be associated with other plant compounds and this association may affect its biodegradation (Sanchez, 2009). One of the main distinctions between cellulose and hemicellulose is that cellulose consists of easily hydrolyzable oligomers while on the other hand; the hemicellulose has branches with short lateral chains consisting of different sugars. In addition, the component lignin is linked to both hemicellulose and cellulose, therefore forming an impenetrable barrier in the plant cell wall. The capability to degrade lignocelluloses efficiently is thought to be associated with a mycelial growth habit that allows the fungus to transport scarce nutrients such as nitrogen and iron, to a distance into the nutrient-poor lignocellulosic substrate that constitutes its carbon source (Hammel, 1997). Fungi have two types of extracellular enzymatic systems: the hydrolytic system, which produces hydrolases that are responsible for polysaccharide degradation; and a

unique oxidative and extracellular ligninolytic system, which degrades lignin and opens phenyl rings (Sanchez, 2009).

2.4 Solid substrate fermentation

Solid substrate fermentation (SSF) is defined as any fermentation process carried out on a solid material in absence of free flowing fluid (Pandey, 2003). Interest in SSF has been increasing because of its importance in productions of enzymes, biopesticides, aroma compounds, biopharmaceuticals, organic acids and other bioactive compounds (Manpreet *et al.*, 2005). Agro industrial residues were also processed using SSF because SSF has lower energy requirement, produce less water, and are environmentally friendly (Manpreet *et al.*, 2005). SSF is considered as an advantageous technique for obtaining edible products with adequate sensory properties and functionality, as well as for improving the nutritional value for raw materials (Longo *et al.*, 2008). SSF has been historically used for cultivation of microorganisms as it approximated the natural growth conditions of most microorganisms (in particular filamentous fungi) (Ugwuanyi *et al.*, 2009).

2.5 Feed grade enzymes

The use of enzymes to increase digestibility and nutritional value of feed has increased, and various depolymerising enzymes have found their way into animal feed as supplement and addictives (Selle *et al.*, 2003). The use of SSF for the production of various feed grade enzymes including xylanase, cellulases and etc is attractive, because in addition to its process engineering advantages, the enzyme may be produced and used (*in situ*) in the feed, in situations where the substrate is also the feed that undergoing (protein and

enzyme) enrichment leading to enhanced digestibility (Karunanandaa *et al.*, 1996; Kang *et al.*, 2004).

2.5.1 Xylanase

Xylanase is one of the enzymes used for feed addictive. Lignocellulosic wastes are known to be composed of cellulose, hemicelluloses and lignin. Hemicelluloses can be classified into either xylans, mannans, arabinogalactans or arabinans based on their sugar backbone composition. Xylanase hydrolyse xylan making cellulose more accessible to cellulase. Xylanase is used as animal feed additives for poultry and pigs, monogastric animals that do not have xylanolytic microflora in their digestive tracts (Longo *et al.*, 2008). Maheswari and Chandra (2000) isolated a mesophilic *Streptomyces cupidosporus* strain which was cultivated under SSF of wheat bran. The crude enzyme preparation, which contain mainly xylanase but also amylase and phytase activities, was applied in predigestion of poultry feed and found to be effective in releasing sugars and soluble phosphorus.

2.5.2 Cellulase

Hydrolases are the main class of enzymes used in monogastric feed, used either to (1) eliminate anti-nutritional factors (ANF) present in grain or vegetables; (2) degrade certain cereal components in order to improve the nutritional value of feed; or (3) to supplement animals' own digestive enzymes (Bhat, 2000). Cellulases are hydrolytic enzymes used for partial hydrolysis of lignocellulosic materials in plant. Lignocelluloses, which are the most abundant, renewable source of energy available, are converted into glucose and fermentable sugars by the action of cellulases. The enzymes cellulases can be applied in the improvement of nutritional quality of animal feed thus improve the performance of

ruminants and monogastrics animals (Bhat, 2000). Besides, the application of cellulases in animal feed biotechnology leads to production and preservation of high quality fodder for ruminants and also improves the quality of grass silage. This is due to the partial hydrolysis of plant cell wall during silage and fodder preservation (Bhat, 2000). Diet of ruminants contains cellulose, hemicelluloses, pectin and also lignin. Enzyme preparations containing high levels of cellulase, hemicellulase and pectinase have been used to improve quality of forages (Bhat, 2000).

2.6 Bioconversion of agricultural waste as animal feed using microorganisms

Aquaculture feeds require high protein level and low fibre. Treatment of agroindustrial waste through SSF leads to significant increase in protein level, and a decrease in cellulose and hemicelluloses (Iluyemi *et al.*, 2006). SSF increases the level of protein and decreased cellulose and hemicelluloses due to the action of xylanases and cellulases (Yang *et al.*, 2001). The use of lignocellulosic waste such as sago waste in animal feeding is constrained by very low protein content and its limited digestibility and palatability to ruminants (Ugwuanyi *et al* ., 2009). However, they may be applied for animal nutrition following protein enrichment by using a variety of micro and macro fungi and bacteria. The production of protein enriched lignocellulosic waste has been associated with the reduction in the content of lignocelluloses (associated with loss of biomass via microbial respiration as CO_2) (Ugwuanyi *et al.*, 2009).

Sherief *et al.*, (2010) has successfully demonstrated the plausibility of using rice straw for animal feed. The results indicate the commercial *P. ostreatus* are able to grow on local rice straw and sawdust residues which in turns caused considerable degradation efficiency for different lignocelluloses fraction by producing a bulk of inducible

lignocellulases (Sherief *et al.*, 2010). The de-lignification, softness, protein richness and free sugar contents after cultivation of *P. ostreatus* increases the availability of rice straw to be used as animal feed.

Iyayi *et al.*, (2004) has demonstrated the improvement of the feeding value of some agroindustrial by-products after solid state fermentation with *Trichoderma viride.* The agroindustrial by-products; brewer's dried grain (BDG), rice bran (RB), palm kernel meal (PKM) and corn bran (CB) undergoes changes in nutrient composition on biodegradation with *Trichoderma viride*; with the increase in protein content when fermented with the fungi for 14 days. In addition, the fibre in the by-products significantly decreased with a corresponding increase of the level of soluble sugars. The results indicate that fungal biodegradation of the agroindustrial by-products can enhance their nutritional status, thus can be used as diet in poultry feed.

According to Nebguide (2008), nutrients of primary concern in feed ingredients includes moisture content, percent crude protein (CP), crude fiber (CF), neutral detergent fiber (NDF), acid detergent fiber (ADF), net energy, calcium, phosporus, copper, zinc and vitamins. A ration which is balanced according to the nutrient needs of livestock leads to livestock productivity. CF is a traditional measure of the content of fibre within a feedstuff, which includes ADF and NDF. NDF measures the cell wall content of a feedstuff. Generally, low value of NDF is desired because NDF increases as forage matures. This is because, when forage matures, the cell wall content increases thus increase the NDF value. ADF on the other hand, is the least digestible plant component which includes celluloses and lignin. ADF values are inversely related to digestibility, so forages with low ADF concentrations are preferred as it is usually higher with energy (Nebguide, 2008). According to Van Saun (2006), higher protein content is usually associated with higher

quality of feed. The amount of protein can be estimated based on the feed sample nitrogen content, by multiplying % N with factor 6.25.

3.0 Materials and Methods

3.1 Microorganism preparation

A pure culture of *Aspergillus niger* PAN1 was maintained as stock culture. It was sub-cultured onto fresh potato dextrose agar plates and incubated for a period of 72-120 hours at 30°C.

A pure culture of *Bacillus amyloliquefaciens* UMAS1002 and *Bacillus licheniformis* P7 was maintained as stock culture. It was grown on nutrient agar at 30°C for a period of 5 days and was stored at 4°C with regular sub culturing after every two weeks.

3.2 Preparation of sago waste as substrate medium

Sago waste (SW) was obtained from local factories, dried at 90°C for overnight. After drying, the sago waste was crushed into smaller pieces by using heavy duty blender, sieved into uniform sizes using 1 mm sieve. After sieving, the sago waste was subjected to alkaline pre-treatment with 4 g L^{-1} NaOH in a weight ratio 1:10 (SW: alkaline solution) for 12 h at room temperature. After alkaline pre-treatment, the excess NaOH was removed by filtering the sago wastes through Muslin cloth and washed extensively with water until the alkaline pH was brought down to pH 7. Next, the treated sago wastes were dried in oven at 90°C for overnight.

3.3 Medium for Solid Substrate Fermentation

Dry treated SW was used as carbon sources. The solid substrate fermentation was conducted in 250 mL flasks with medium of treated ground SW. The substrate moisture

was adjusted to 80% by adding mineral salt medium with the following composition in gram per litre: 0.2 % yeast extract; 0.1 % KH_2PO_4 and 0.5% $MgSO_4$.

3.3.1 Size of inoculums

Inoculums were prepared in a 250 mL conical flasks containing 10 g of SW as substrate. By using 250 mL flasks, fermentation medium was added and followed by inoculating the flasks with three plugs of *A. niger* PAN1 on Day 1. The flasks were then inoculated with 0.5% (v/v) of *Bacillus amyloliquefaciens* UMAS1002 and 0.5% (v/v) *Bacillus licheniformis* P7 suspension on Day 3. Experiments are done in duplicate and the average values are calculated. For each interval of 48 h, a control was also prepared, by which the substrate moisture were also increased to 80 %, with the absence of inoculums, throughout the period of 6 days of fermentation.

3.4 Xylanase and cellulase production under SSF

The SSF for the production of xylanase and cellulase was carried out as described in Section 3.3. At time intervals of 48 hours, 1 ml broth was harvested and separated by ultracentrifugation and crude filtrate was assayed for xylanase and cellulase activity (Khairnar *et al.,* 2009). Xylanase and cellulase were assayed using Di-nitrosalicyclic (DNS) method (Miller, 1959). Xylanase activity was determined by using xylan immersed in 0.1M sodium citrate buffer, pH 5.5. Xylanase activities were expressed in terms of international unit (IU). One IU was defined as the amount of enzyme required to release 1 μmol xylose equivalents in 1 mL of enzyme solution in one minute. Enzyme activities were expressed as IU mL^{-1}. The colour absorption was then determined using UV visible

spectrophotometer at 550 nm. For cellulase, the activity was determined using 1% CMC immersed in 0.5M sodium acetate buffer, pH 5. One unit of cellulase activity is defined as the amount of enzyme required to liberate 1 μmol of glucose equivalents in 1 mL solution in one minute. Enzyme activities were expressed as IU mL^{-1}. The colour absorption was then determined using UV visible spectrophotometer at 540 nm.

3.5 Protein determination

Soluble protein in the enzyme solution was determined by using Bradford method (1976) by measuring the optical density of the colour developed at 595 nm using a spectrophotometer. The μg of protein was estimated based on BSA as standard curve.

3.6 Total N determination using micro-Kjeldahl method

The Kjeldahl (1883) method was a standard method used to determine the nitrogen content of organic and inorganic substances. Micro-Kjeldahl method was used for analysis of nitrogenous compound in relatively small amount, samples with total nitrogen content in the range of 1 to a few milligrams. The Kjeldahl method was the standard method of nitrogen determination which involves there basic steps: 1) Digestion - the decomposition of nitrogen in organic samples utilizing a concentrated acid solution. This was accomplished by boiling a homogeneous sample in concentrated sulfuric acid. The end result was an ammonium sulfate solution; 2) Distillation - adding excess base to the acid digestion mixture to convert NH_4^+ to NH_3, followed by boiling and condensation of the NH_3 gas in a receiving solution and 3) Titration - to quantify the amount of ammonia in the

receiving solution. The amount of nitrogen in a sample was calculated from the quantified amount of ammonia ions in the receiving solution.

3.7 Crude protein estimation

Crude protein was derived through a calculation using nitrogen value obtained. Crude protein was estimated by multiplying total nitrogen N weight obtained with factor 6.25. Multiplying total nitrogen value with 6.25 gives the crude protein content which also includes non protein nitrogen. In order to get true protein content, non-protein nitrogen was deducted from the total nitrogen and then was multiplied with the factor. A factor of 6.25 is used to convert total nitrogen in animal feeds into crude protein.

3.8 Determination of residual lignin, cellulose and hemicelluloses

3.8.1 Washing and Digestion Stage

The AOAC (1990) method was modified for the determination of residual lignin, cellulose and hemicelluloses. Washing stage is the first stage done in the determination of residual lignin. Firstly, 1.0 g of the sample was mixed with double distilled water and shaked in a rotary shaker at 120 rpm for 1 hour. The double distilled water was then poured out and ethanol was then added into the sample. The mixture was shaked again for 1 h on a rotary shaker at shaking speed of 120 rpm. The ethanol was then poured out and acetone was added. This mixture was then shaked again for another 1 h on a rotary shaker at 120 rpm. Excess acetone was then poured out and the resulting washed material was left for overnight under a fume hood. The next day, digestion stage was carried out. Approximately, 0.4 g of the washed sample were weighed and digested with 70% (v/v)